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Characterization of two monoclonal antibodies (UCL4D12 and UCL3D3) that discriminate between human mantle zone and marginal zone B cells

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SUMMARY

Two new monoclonal antibodies (MoAbs), UCL3D3 and UCL4D12 were obtained following immunization with follicular lymphoma (UCL3D3) or low-grade primary B cell gastric lymphoma cells (UCL4D12). In normal splenic white pulp, tonsil and small intestinal Peyer's patches, UCL4D12 recognizes marginal zone B cells and a subpopulation of follicle centre cells, whereas mantle zone B cells are UCL4D12 negative. In contrast, UCL3D3 recognizes mantle zone B cells and follicular dendritic cells, but not marginal zone B cells or follicle centre B cells. Double-immunofluorescence studies showed that in the splenic white pulp, these antibodies stain reciprocally. The majority of UCL3D3$^+$ cells are sIgM$^+$ and sIgD$^+$ whereas a higher proportion of UCL4D12$^+$ cells express surface IgM (sIgM) but not surface IgD (sIgD). Less than 10% of splenic B cells express both 3D3 and 4D12 antigens. None of the cell lines tested expressed either antigen. Functional studies showed that both antigens play a role in B cell activation as the MoAbs increase the mitogenic effect of *Staphylococcus aureus* Cowan I on tonsil B cells. This effect was maximal at 72 h in culture. TPA activation was reduced, and no effect was observed with anti-immunoglobulin (anti μ) or CDw40 (G28.5). UCL3D3 and UCL4D12 did not show any stimulatory effect on their own. Biochemical studies show that both MoAbs recognize proteins of 80–90 kD under reducing conditions. These two MoAbs appear to recognize new B cell surface antigens which may be useful for identifying subpopulations of B cells.

Keywords monoclonal antibodies B cells marginal zones mantle zones

INTRODUCTION

The introduction of monoclonal antibodies (MoAbs) to leucocyte surface antigens has allowed the identification of many subpopulations in normal tissues and their analogues in malignant tissues (Reinherz *et al.*, 1985; MacMichael *et al.*, 1987; Knapp, 1989). However, the lineage relationship of human B cell subpopulations remains unresolved.

Three major populations of B lymphocytes are recognizable in tissue sections. The cells of the mantle zone are sIgM$^+$ and sIgD$^+$ as well as expressing *pan*-B cell antigens (CD19, CD20) characteristic of mature B cells. It has been shown that these cells belong to a recirculating pool of unprimed B cells which move between peripheral blood (PB) and the mantle zones and primary follicles of lymphoid tissues (Gray *et al.*, 1982). It is thought that mantle zone lymphocytes give rise, following antigenic stimulation, to the second anatomically recognizable population, the follicle centre cells. Follicle centres contain rapidly dividing cells that express sIgM but not IgD. They may be involved in the transport of antigens (Gray *et al.*, 1984). It is likely that somatic mutation events leading to production of high affinity antibodies take place in this site (MacLennan & Gray, 1986).

The third population of B cells is that of the splenic marginal zone which contains a mixture of cells at intermediate stages between small lymphocytes and plasmablasts, as well as macrophages (Humphrey, 1981). These B cells express sIgM but not sIgD and have avid Fc and C3 receptors (Gray *et al.*, 1982, Liu, Oldfield & MacLennan, 1988). Two functions have been ascribed to marginal zone cells. In earlier work it was shown that these cells are important in immune responses to thymus-independent (TI2) antigens (Humphrey, 1981), a result in agreement with the observation that splenectomized individuals who are depleted of marginal zone cells, respond poorly to thymus independent antigens (Amlot & Hayes, 1985). More recently extensive studies in the rat have led to the suggestion that the marginal zone contains memory B cells (MacLennan *et al.*, 1982; Kumararatne & MacLennan, 1981; Zhang *et al.*,

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1988). It is unclear how these two functional populations of B cells are related to each other and to follicle centre and mantle zone cells particularly in view of data suggesting that primary and secondary B cells may be independent cell lineages (Linton & Klinman, 1989).

Much of our understanding of human lymphocyte function is derived from *in vitro* experiments. A large number of B cell surface antigens have been identified with MoAbs and characterized biochemically and at the DNA level (Reinherz *et al.*, 1985; Kikutani *et al.*, 1986; MacMichael *et al.*, 1987; Knapp, 1989). Many of these MoAbs have also been used to study the mechanisms of B cell activation and differentiation, often in co-stimulation experiments with other agents such as anti-IgM, *Staphylococcus aureus* Cowan I or phorbol esters (Kishimoto *et al.*, 1975; Parker, 1975; De Franco *et al.*, 1982; Kehrl, Muraguchi & Fauci, 1984; De Franco, Raveche & Paul, 1985; Clark & Ledbetter, 1989).

Here we examined the tissue distribution and function of the antigens identified by two new MoAbs, UCL3D3 and UCL4D12. These identify mantle and marginal zone B cells, respectively, and may therefore be useful in resolving some of the uncertainties relating to the function and lineage relationships between mantle and marginal zone B cells and their relation to the follicle centre.

MATERIALS AND METHODS

Production of monoclonal antibodies

Suspensions of lymphoid cells were frozen down in aliquots of 5×10^6 cells for subsequent immunizations. BALB/c mice were immunized (5×10^6 cells/immunization) with cells teased from either a follicular lymphoma (UCL3D3) or a low grade lymphoma of mucosal-associated lymphoid tissue (MALT) (UCL4D12) 17 and 3 days prior to fusion. Spleen cells were fused with the NSO myeloma cell line using previously described techniques (Waldmann, 1986). Supernatants were screened on frozen sections of normal spleen using indirect immunoperoxidase (Isaacson & Wright, 1983). The isotypes of the antibodies to be studied in detail were determined using double immunodiffusion in agarose gel and found to be IgM (UCL3D3) and IgG2b (UCL4D12).

Cell preparation

Mononuclear cells were isolated from normal peripheral blood, tonsil or spleen. Cells were teased out from tissue and centrifuged on Ficoll/Hypaque (Pharmacia, Uppsala, Sweden; $\rho = 1{\cdot}077$ kg/*l*) at 2500 rev/min for 25 min. The T cells were removed by rosetting with 2-aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes for 1 h at 4°C. Further purification was done using a CD3 antibody (UCHT1) (Beverley & Callard, 1981) and sheep anti-mouse IgG coupled to magnetic beads (Dynabeads, Dynatech). The remaining T cells were removed by attraction with a magnet (Dynatech). The final preparation of E^- cells contained more than 90% of B cells as tested with a commercial *pan*-B antibody (Leu 16, Becton Dickinson). Spleen cells were used fresh or frozen in liquid nitrogen for subsequent staining. All cell lines were grown in RPMI 1640 medium (GIBCO) containing 10% fetal calf serum (FCS) and 0·1 mg/ml streptomycin.

Immunocytochemistry

Reactivity of antibodies UCL3D3 and UCL4D12 with normal tissues (tonsil, spleen and Peyer's patch) was tested using the indirect immunoperoxidase method. Frozen sections cut at 8 μm were acetone-fixed and incubated in primary MoAb for 60 min. The *pan*-B cell MoAb CD22 (Dako) was included in this study for comparison with UCL3D3 and UCL4D12 hybridoma supernatants. Sections were then incubated in peroxidase-conjugated rabbit anti-mouse antiserum (Dako) and peroxidase activity was visualized using the diaminobenzidene reagent. For double immunofluorescence, sections of spleen cut at 8 μm were fixed in acetone for 30 min and subsequently incubated for 45 min in a mixture of equal volumes of UCL3D3 and UCL4D12. Sections were then incubated in a mixture of goat anti-mouse IgG conjugated to FITC and goat anti-mouse IgM conjugated to TRITC (Southern Biotechnology). Sections were photographed using a double exposure at the appropriate wavelengths. The distribution of green ($UCL4D12^+$, $UCL3D3^-$), red ($UCL4D12^-$, $UCL3D3^+$) and yellow ($UCL4D12^+$, $UCL3D3^+$) cells was then determined.

Purification of monoclonal antibodies

Purified UCL4D12 was obtained from ascites fluid which was diluted 2/1 with 0·9% NaCl and precipitated with 45% saturated ammonium sulphate at room temperature for 30 min. The precipitate was spun at 2500 rev/min for 15 min and washed once in 45% ammonium sulphate. The pellet was resuspended in a small volume of phosphate-buffered saline (PBS) and dialysed overnight with one change, against 0·1 M phosphate-buffer pH 8. The solution was run through a protein A column and the antibody eluted with 0·1 M citrate buffer pH 3·5. Purified UCL-3D3 was obtained using the procedure described by Neoh (Neoh *et al.*, 1986). The concentrations of UCL-3D3 and UCL-4D12 were calculated according to the optical density at 280 nm ($OD_{280} = 1{\cdot}4$ equivalent to 1 mg/ml protein).

Preparation of antibody-coupled beads

UCL-3D3 and UCL-4D12 were coupled to cyanogen bromide activated Sepharose 4B beads which had been washed on a sintered glass filter with 1 mM HCl, then with coupling buffer (0·1 M $NaHCO_3$, pH 8·3). The MoAbs were diluted in coupling buffer containing 0·5 M NaCl and 2 mg/ml of antibodies were mixed with 1 ml of beads. The mixture was rotated at 4°C overnight. Excess of antibody was washed off with coupling buffer. The remaining beads were saturated with 0·1 M Tris buffer pH 8, then subjected to three cycles of washes. Each cycle was carried out in 0·1 M acetate buffer pH 4 containing 0·5 M NaCl followed by a wash with 0·1 M Tris/HCl buffer, pH 8, containing 0·5 M NaCl.

Biotinylation of UCL-3D3 and UCL-4D12

One milligram of MoAbs (1 mg/ml in NaHCO pH 8·6) was mixed with 30, 60 and 120 μg of Biotin-X-NHS (biotinyl-ε-amino-caproic acid *N*-hydroxysuccinimide ester, Calbiochem) dissolved at 1 mg/ml in dimethyl sulphoxide. The mixture was incubated at room temperature for 4 h. The biotinylated antibodies were dialysed overnight with one change against PBS containing 0·1% azide.

Antibodies

The following antibodies were used: UCL3D3 (IgM) and UCL4D12 (IgG2b) purified MoAbs, biotin or FITC conjugated; monoclonal anti-human IgA1, IgA2 (IgG1 isotype, Oxoid, Bedford, UK). UCHT1 (CD3, IgG1 isotype) culture supernatant used undiluted (Beverley & Callard, 1981); Leu 16 (CD20, Becton Dickinson); anti-mouse IgG1, FITC-conjugated (Dako); polyclonal rabbit anti-mouse immunoglobulin, FITC conjugated (Dako); polyclonal rabbit anti-human IgM, IgD, IgG, FITC conjugated (Dako); and streptavidin PE (Becton Dickinson).

Single and dual immunofluorescence staining and analysis

For single-colour staining, 5×10^5 cells/well were incubated for 30 min at 4°C in round-bottomed microtitre plates, washed three times with MEM containing 5% FCS and then incubated with rabbit anti-mouse immunoglobulin. For double staining three protocols were used: (i) cells were first incubated and washed (as above) with polyclonal rabbit anti-human IgM, IgD or IgG, FITC-conjugated, followed by biotinylated UCL3D3 or UCL4D12 and streptavidin PE; (ii) cells were incubated successively with monoclonal anti-A1 or anti-A2, anti-mouse IgG1–FITC, biotinylated UCL3D3 or UCL4D12 and finally streptavidin PE; and (iii) in some experiments, cells were first incubated with UCL4D12–FITC followed by biotinylated UCL3D3 and streptavidin PE. Stained cells were analysed by flow cytofluorometry on a FACScan (Becton Dickinson).

Cell lines

The following cell lines were tested for UCL3D3 and UCL4D12 expression: T lymphoblastoid, CEM, HUT-78, JM, J6 and MOLT4; B lymphoblastoid, GK, HFB-1, WMPT, L4, L6, Daudi, BL74 and BL29; Hodgkins derived, Hs445; myeloid, U937, HL60 and K562; non-lymphoid UCH10 and a $CD23^+$ L cell transfectant (gift from Dr T. Kishimoto). Eight $CD5^+$ EBV-B cell lines were kindly tested for us by Dr A. Shutte (Academische Ziekenhuis, Utrecht).

Cell culture conditions and activation

Cell lines and fresh B cells from tonsils were cultured in HEPES-buffered RPMI 1640 medium (GIBCO) containing 10% FCS. For lymphocyte activation, formalinized *St. aureus* Cowan I or TPA were used at 50 μg/ml and 10 ng/ml, respectively. Tonsil B cells at a concentration of $7{\cdot}5 \times 10^5$/ml were cultured in 200 μl of medium in flat-bottomed microtitre plates for 72 h at 37°C in an atmosphere of 5% CO_2 in air (six wells for each mitogen concentration). After 64 h of incubation, 1 μCi of ^{3}H-thymidine (Amersham) was added to each well and the cells were further incubated for 8 h at 37°C. Cells were harvested onto filter paper discs (Whatman) using a Dynatech automash II cell harvester. The discs were dried and placed in 2 ml of scintillation fluid and counted on a Beckman LS 1800 Liquid scintillation counter.

Iodination of B cells

Tonsil B cells were prepared as described earlier. Cells (4×10^7) were washed in PBS and labelled with 1 mCi ^{125}I using the lactoperoxidase method (Walsh & Crumpton, 1977). At the end of the labelling, the cells were washed in PBS and the pellet dissolved in lysis buffer (3·3% Brij 96 and 1·7% Brij 99), 10 mM Tris, pH 7·4, 1 mM EDTA, 150 mM NaCl) containing 1 mg/ml BSA and 1 mM phenylmethane sulphonyl fluoride (PMSF) for 30 min. Immunoprecipitations were carried out as described elsewhere (Owen, Kissonerghis & Lodish, 1980) using UCL3D3 and UCL4D12 coupled to Sepharose beads. The immunoprecipitated proteins were removed from the beads by boiling in sample buffer. The samples were loaded on a 5–12% polyacrylamide gradient gel using the Laemmli method (Laemmli, 1970).

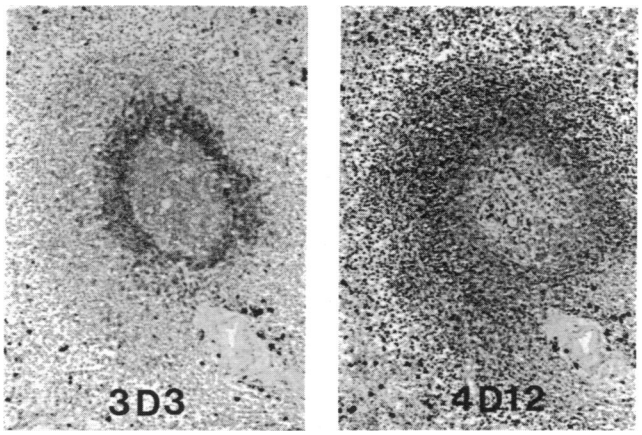

Fig. 1. Serial sections of splenic white pulp stained using immunoperoxidase with monoclonal antibodies UCL3D3 and UCL4D12. UCL4D12 recognizes marginal zone B cells and a subpopulation of follicle centre cells. Mantle zone B cells are UCL4D12 negative. UCL3D3 recognizes mantle zone B cells and follicular dendritic cells. Marginal zone B cells and follicle centre B cells are UCL3D3 negative.

RESULTS

Immunohistochemistry and cell staining

The screening procedure revealed two MoAbs UCL3D3 and UCL4D12 which recognized subpopulations of B cells in normal spleen. In normal lymph node, tonsil and spleen, UCL3D3 recognized mantle zone cells and follicular dendritic cells (Fig. 1). It did not stain follicle centre B cells or splenic marginal zone cells. In contrast, splenic marginal zone B cells were strongly $UCL4D12^+$ whereas mantle zone B cells in lymph node, tonsil and spleen were largely $UCL4D12^-$ (Fig. 1). MoAb UCL4D12 recognized a subset of follicle centre cells in lymph node, tonsil and spleen which were concentrated in the light zone of the follicle centre abutting on the broadest aspect of the mantle zone. Double immunofluorescence showed that $UCL4D12^+$ and $UCL3D3^+$ populations were mutually exclusive in splenic white pulp (Fig. 2). The small intestinal Peyer's patch studied contained a small $UCL3D3^+$, $UCL4D12^-$ mantle zone surrounded by a more extensive $UCL3D3^-$, $UCL4D12^+$ marginal zone.

UCL3D3, UCL4D12 and surface immunoglobulin expression

Single staining of tonsil and spleen B cells shows that UCL3D3 identifies 20–40% of tonsil B cells and 50–60% of spleen B cells. UCL4D12 stains 5% of tonsil B cells and 15–30% of spleen B cells. In normal PBL, up to 90% of B cells are $UCL3D3^+$ cells and less than 2% $UCL4D12^+$ cells.

Tonsil B cells were cultured for 3 days and samples were removed after 24, 48, 72 h and stained with UCL3D3 and UCL4D12. After 72 h, only 2–5% of B cells were positive for UCL4D12 and UCL3D3, showing that 3D3 and 4D12 antigens are lost quickly from the cell surface. The addition of mitogens

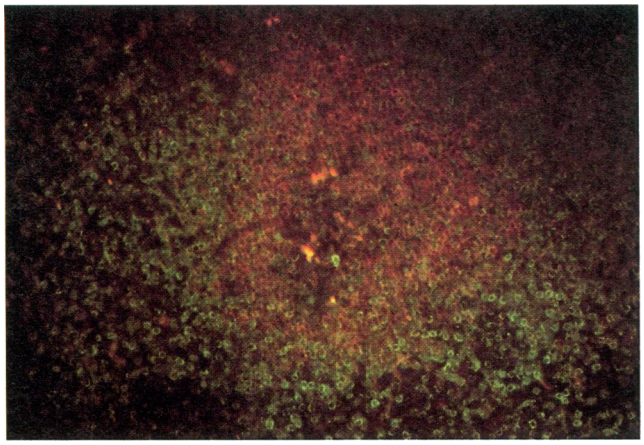

Fig. 2. Section of spleen stained using double immunofluorescence with monoclonal antibodies UCL4D12 (green) and UCL3D3 (red). The absence of cells staining yellow indicates that in the splenic white pulp, B cells express either UCL4D12 or UCL3D3, or as indicated by dark areas of the follicle centre, stain with neither.

like SAC or TPA to the medium enhanced the rate of decrease of 3D3 and 4D12 antigens on the cell surface (data not shown). In no experiment was 3D3 or 4D12 antigen expression induced by mitogen activation. In addition, 20 different cultured human cell lines were stained, including B and T lymphoid cells, myeloid, erythroid and Hodgkin's lines, and carcinomas. None were consistently positive with either MoAb.

Dual fluorescence analysis of spleen and tonsil B cells showed that 90% of 3D3-expressing cells are $sIgM^+$ and 85% are $sIgD^+$ (Fig. 3), a small population express IgA1 and IgA2 (Table 1). A low proportion of $3D3^+$ cells do not express either sIgM or sIgD. In contrast, the majority of $4D12^+$ cells express sIgM, but the expression of sIgD, although differing in tonsil and spleen, is lower than on $3D3^+$ cells. A significant proportion of $UCL4D12^+$ cells express IgA1, IgA2, or IgG (36%, 28% and 23%, respectively). Double staining of spleen cells in suspension shows that only about 7% of B cells express, both weakly, 3D3 and 4D12 antigens.

Gel electrophoresis results

The molecular weights of 3D3 and 4D12 antigens were examined by immunoprecipitation and electrophoresis on a 5–12% polyacrylamide gradient gel (Fig. 4). Diffuse protein bands at 140–180 kD for UCL3D3 and 120–160 kD for UCL4D12 were observed under non-reducing conditions. An extra band at 52 kD is also shown for 4D12 antigen. Under reducing conditions, 3D3 antigen gives a broad band which on less-exposed gels is composed of two polypeptides at around 72–90 kD. 4D12 antigen has a major band at 70–80 kD. In some cases two bands could be seen in that size range on a 10% polyacrylamide gel.

B cell activation

Tonsil B cells were cultured with SAC and purified UCL3D3 or UCL4D12. Our results indicate that both MoAbs enhance the response to SAC at concentrations up to 50 μg/ml of MoAbs (Fig. 5) when an optimal concentration of 50 μg/ml SAC was used. UCL4D12 always showed higher costimulation effect with SAC (50% for UCL4D12 and 35% for UCL3D3). At concentrations of MoAbs greater than 50 μg/ml, an inhibitory effect was observed. In contrast, TPA activation of B cells was partially inhibited by UCL3D3 and UCL4D12. This inhibitory effect was about 30% (Fig. 6). Although a relatively small effect, the inhibition was consistent in several repeat experiments. We also investigated the effect of UCL3D3 and UCL4D12 on the response to cross-linking by anti-IgM (anti-μ). No effect was observed (data not shown). Some MoAbs can induce proliferation when combined with CDw40 (G28·5) but this effect was not observed for UCL3D3 and UCL4D12 (data not shown).

DISCUSSION

We have described the tissue and cellular distribution and some functional properties of two new MoAbs, UCL3D3 and UCL4D12. Anatomically UCL3D3 recognizes B cells in the mantle zone of lymph nodes and spleen as well as follicular dendritic cells while UCL4D12 stains splenic marginal zone cells and a subset of follicular centre cells. Double immunofluorescence studies of splenic white pulp showed that B cell populations expressing these two antigens were nearly mutually exclusive. Peyer's patches are difficult to study because well-orientated frozen sections are difficult to prepare. We were able to study one specimen, however, which showed that as in the spleen, the mantle zone was $UCL3D3^+$, $UCL4D12^-$ in comparison with the surrounding, more extensive marginal zone B cells which were $UCL3D3^-$, $UCL4D12^+$. As may be predicted from this tissue distribution, $UCL3D3^+$ cells are mainly $sIgM^+sIgD^+$, while $UCL4D12^+$ cells are also mainly $sIgM^+$ but a higher proportion lack sIgD. A significant proportion of UCL4D12 cells express sIgA or sIgG. More than 90% of blood B cells express UCL3D3 and a few double-positive cells can be found in any tissue. In tonsil cell suspensions a significant proportion of B cells lacks both antigens. The only cells of B cell lineage in sections of normal and reactive lymphoid tissues which were not stained by either UCL4D12 or UCL3D3 were plasma cells and B cells in the dark zone of the follicle centre where the centroblasts are concentrated, but the phenotype and function of this double-negative population remains to be investigated in detail.

Thus these two MoAbs appear to recognize two functionally distinct populations, the mantle zone B cells which re-circulate through blood and lymphoid organs and initiate primary antibody responses, and the marginal zone population of sessile cells. The function of the latter is controversial since both responses to thymus-independent antigens and memory function have been ascribed to it (Humphrey, 1981; MacLennan *et al.*, 1982). Removal of the spleen, which would be expected to remove most marginal zone B cells, does depress responses to thymus-independent antigens (Amlot & Hayes, 1985). In support of the second possibility, data on recall responses to influenza and herpes zoster viruses show that memory B cells do not circulate for long periods in blood but can be found in the spleen (Callard *et al.*, 1982; Souhami, Babbage & Sigfusson, 1983), a finding in accord with the low number of UCL4D12 (memory/marginal zone) cells in blood and high number in spleen. In addition, studies in rats have shown that splenic marginal zone cells are responsible for immune responses to thymus-independent antigens (MacLennan *et al.*, 1982). However, it has been shown that memory B cells to thymus-dependent antigens which arise from antigen-dependent pro-

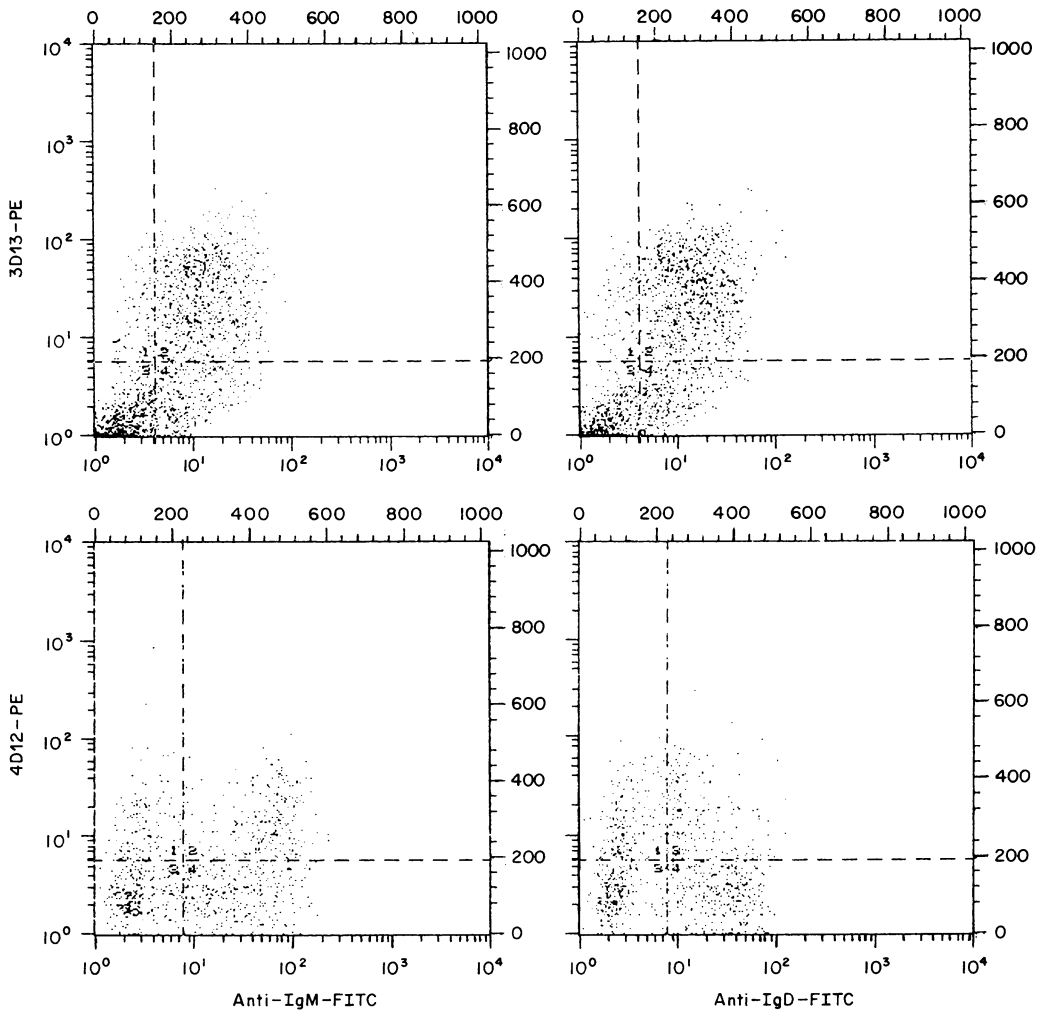

Fig. 3. Double-staining pattern of UCL3D3 and UCL4D12 with sIgM and sIgD on spleen cells. Analysis was carried out as described in Materials and Methods. FACS analysis of UCL3D3 (top) and UCL4D12 (bottom) against anti-IgM (left), anti-IgD (right).

Table 1. Results of double-staining of UCL3D3 or UCL4D12 with sIg

	UCL3D3		UCL4D12	
Immunoglobulins	Spleen	Tonsils	Spleen	Tonsils
sIgM	90	85	75	40
sIgD	85	82	48	12
sIgA1	13	—	36	—
sIgA2	20	—	28	—
sIgG	4	—	23	—

Mean (%) of three experiments for sIgA1, sIgA2, sIgG and four experiments for sIgM and sIgD.

liferation in the follicle centre also reside in the rat splenic marginal zone (Liu *et al.*, 1988). Whether human marginal zone B cells are specific for thymus-independent antigens or are memory B cells, they are united by common morphological characteristics and reactivity with UCL4D12.

UCL4D12 has an interesting pattern of reactivity in the follicle centre: it recognizes only a subpopulation of medium-sized cells in the light zone where the centrocytes are known to be concentrated. This raises an interesting question of the relationship between the follicle centre and mantle and marginal zone B cells. Follicle centres arise from primary B cell follicles which, like the mantle are IgD^+ (Stein, Gerdes & Mason, 1982). It has also been shown that rat marginal zone B cells, following antigenic stimulation, may migrate into the follicle centre (Gray *et al.*, 1984). The inter-relationship between these B cell compartments is reflected in the behaviour of the centrocyte-like (CCL) cells in MALT lymphomas. These cells, like their probable analogues the marginal zone B cells, show a tendency to infiltrate follicle centres (Myhre & Isaacson, 1987; Isaacson *et al.*, 1989). We have not yet investigated the functional properties of separated $UCL3D3^+$ and $4D12^+$ cells but it is likely that the latter may contain memory as well as responding to pokeweed mitogen, since the B cells which respond to this mitogen have been shown to be $sIgM^+$, $sIgD^-$ (Kuritani & Cooper, 1982).

SDS-PAGE analysis of labelled surface proteins of tonsil B cells shows that UCL3D3 and UCL4D12 antigens are high molecular weight proteins giving under non-reducing conditions, diffuse bands at 140–180 kD and 120–160 kD for 3D3 and 4D12 antigens, respectively. On less exposed gels they appear

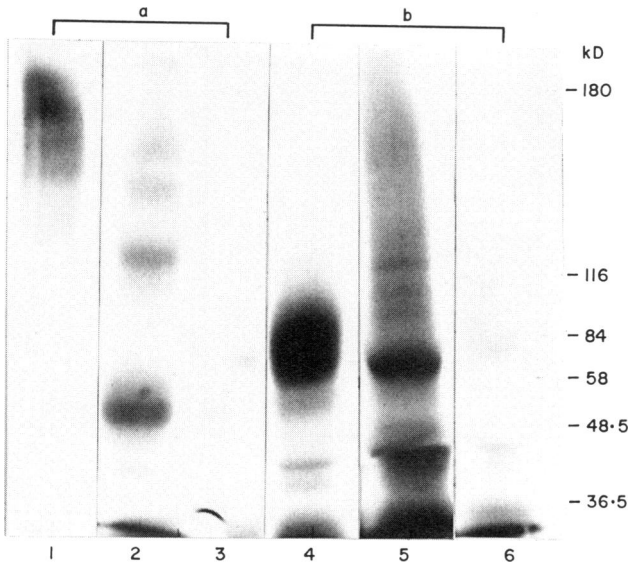

Fig. 4. Gel electrophoresis of 3D3 and 4D12 antigens. On a 5–12% polyacrylamide gradient gel at constant voltage (40 V) for 16 h; (a) non-reducing conditions; (b) reducing conditions. Lanes 1 and 4, 3D3 antigen; 2 and 5, 4D12 antigen; 3 and 6, controls.

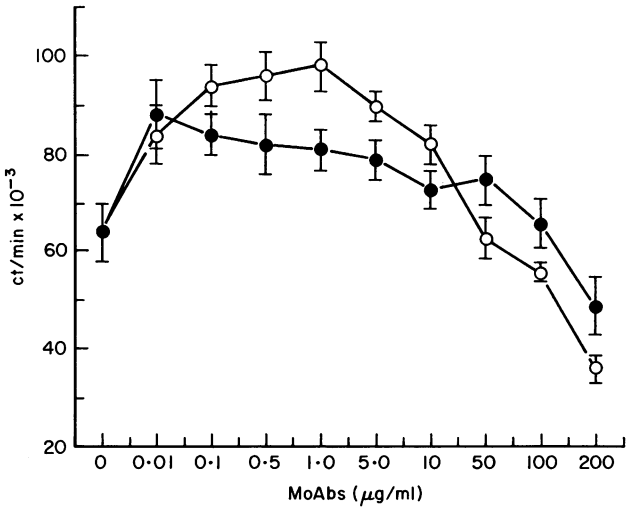

Fig. 5. Co-stimulation of UCL3D3 (●) and UCL4D12 (○) with 0·005% SAC on tonsil B cells. Effect of different concentrations of UCL3D3 And UCL4D12. Cell culture and activation were as described in Materials and Methods. Bars are s.e.m. for each concentration of MoAbs.

to have several bands, which may indicate the presence of disulphide bonds within the protein molecules. An extra band at 52 kD is observed for 4D12 antigen. Under reducing conditions, 3D3 antigen gives two bands at around 90–72 kD. UCL4D12 gives a band at around 80 kD. These results suggest that UCL3D3 and UCL4D12 recognize antigen molecules of high molecular weight which are composed of polypeptides linked by disulphide bonds. Although many anti-B cell MoAbs have been reported at the International Workshops on Human Leucocytes

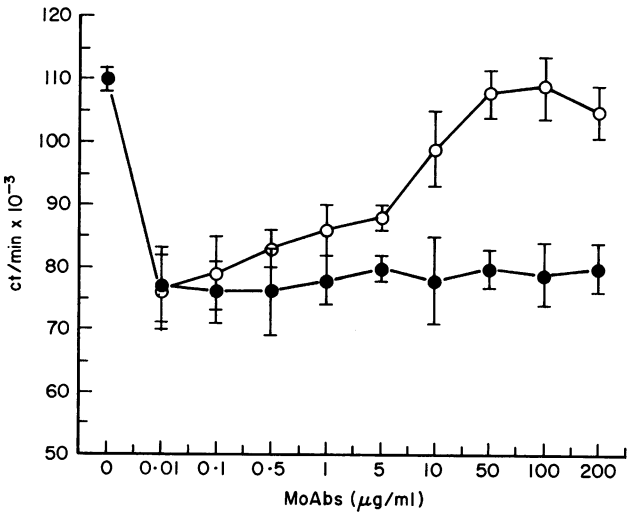

Fig. 6. Inhibition of TPA activation by UCL3D3 (●) and UCL4D12 (○). Effect of different concentrations of MoAbs; TPA concentration was 10 ng/ml.

Differentiation Antigens (Reinherz *et al.*, 1985; MacMichael *et al.*, 1987; Knapp, 1989) to react with subpopulations of B cells, the tissue distribution and biochemical properties of UCL-3D3 and 4D12 appear to distinguish them from the majority of these. However, MoAb UCL3D3 presents several similarities with another MoAb, UCHB1, that has been isolated and characterized recently in our laboratory (Armitage, Rowe & Beverley, 1988). Both stain mantle zone B cells and costimulate with SAC and both are lost after *in vitro* activation. The main differences are that UCHB1 increases TPA activation and does not stain CLL cells. In the 4th Leucocyte Workshop (Knapp, 1989) UCHB1 was reported to be anti-human IgM. In recent experiments, using an ELISA we have confirmed that UCHB1 does bind to IgM but in the same assay neither UCL3D3 nor UCL4D12 showed any reactivity. In addition, blocking experiments on tissue sections using polyclonal anti-human IgA1, IgA2, IgD, IgG and IgM demonstrate that these MoAbs do not detect any of the immunoglobulin classes tested (unpublished data).

UCL3D3 and UCL4D12 MoAbs increase SAC activation of tonsil B cells. This costimulatory effect is not great, particularly for UCL3D3, but is reproducible. However, since only a proportion of B cells express these antigens the effects, particularly for UCL4D12, are more impressive than they appear at first. Neither MoAb has any activating effect alone or when coupled to Sepharose beads. The effect of these two MoAbs on SAC stimulation could be due either to an increase in the stimulatory effect of SAC on tonsil B cells or a costimulatory effect of SAC and the MoAbs on a subset of B cells which cannot be activated by SAC on its own. When UCL3D3 and UCL4D12 were added at different times (24, 48, 72 h) following SAC activation, we did not observe any substantial difference in the costimulatory effect. These results suggest that these MoAbs increase the mitogenic effect of SAC on tonsil B cells that are already stimulated with SAC alone. The way UCL3D3 and UCL4D12 stimulate B cells is not yet clear but cross-linking is unlikely to be important as they do not show any effect on tonsil B cells when coupled to Sepharose beads. The stimulatory effects of UCL3D3 and UCL4D12 appear specific to SAC, since

they inhibit TPA activation and have no effect with CDw40 (G28.5) and anti-μ which also activate some resting B cells. None of the cell lines tested by FACS analysis showed reactivity with UCL3D3 and UCL4D12, which may mean that the corresponding antigens are expressed only on resting B cells. In support of this, 3D3 and 4D12 antigens are lost rapidly from the surface of tonsil B cells and this loss is faster when the cells are cultured in the presence of activating agents that drive the cells into cycle (SAC, TPA). Alternatively, these antigens may be expressed only in particular tissue micro-environments that are disrupted when cells are cultured *in vitro*.

The studies we have carried out to characterize these two MoAbs show that they differ from previously described clusters of differentiation. They are able to discriminate between different subpopulations of B cells in the splenic marginal and mantle zones and will be useful in studying the phenotype and distribution of these cell populations. UCL4D12 is of particular interest as it is one of the few B cell MoAbs that recognize specifically human splenic marginal zones. Future experiments will investigate the function of these two cell populations and in particular test the view that UCL4D12$^+$ cells contain memory for thymus-dependent protein antigens.

REFERENCES

AMLOT, P.L. & HAYES, A.E. (1985) Impaired human antibody response to the thymus-independent antigen DNP ficoll, after splenectomy: implification for post-splenectomy infections. *Lancet*, **i,** 1008.

ARMITAGE, R.J., ROWE, D.J. & BEVERLEY, P.C.L. (1988) A new antigen identified by the monoclonal antibody UCHB1 delivers a costimulatory signal to a subset of human B cells. *Eur. J. Immunol.* **18,** 67.

BEVERLEY, P.C.L. & CALLARD, R.E. (1981) Distinctive functional characteristics of human 'T' lymphocytes defined by E rosetting or a monoclonal anti-T cell antibody. *Eur. J. Immunol.* **11,** 329.

CALLARD, R.E., MCCAUGHAN, G.W., BABBAGE, J. & SOUHAMI, R.L. (1982) Specific in vitro antibody responses by human blood lymphocytes: apparent non-responsiveness of PBL is due to lack of recirculating memory B cells. *J. Immunol.* **129,** 153.

CLARK, E.A. & LEDBETTER, J.A. (1989) Structure, function, and genetics of human B-cell associated surface molecules. *Adv. Cancer Res.* **82,** 81.

DE FRANCO, A.L., RAVECHE, E.S. & PAUL, W.E. (1985) Separate control of B lymphocyte early activation and proliferation in response to anti-IgM antibodies. *J. Immunol.* **135,** 87.

DEFRANCO, A.L., RAVECHE, E.S., ASOFSKY, R. & PAUL, W.E. (1982) Frequency of B lymphocytes responsive to anti-immunoglobulin. *J. exp. Med.* **155,** 1523.

GRAY, D., KUMARARATNE, D.S., LORTAN, J., KHAN, M. & MACLENNAN, I.C.M. (1984) Relation of intra-splenic migration of marginal zone B cells to antigen localization on the follicular dendritic cells. *Immunology*, **52,** 659.

GRAY, D., MACLENNAN, I.C.M., BAZIN, H. & KHAN, M. (1982) Migrant μ^+ δ^+ and static $\mu+\delta$-B lymphocyte subsets. *Eur. J. Immunol.* **12,** 564.

HUMPHREY, J.H. (1981) Tolerogenic or immunogenic activity of hapten-conjugated polysaccharides correlated with cellular localization. *Eur. J. Immunol.* **11,** 212.

ISAACSON, P.G. & WRIGHT, D.H. (1983) Immunocytochemistry of lymphoreticular tumours. In *Immunocytochemistry. Practical Applications in Pathology and Biology* (ed. by J. M. Polak & S. Van Noorden) p. 249. John Wright & Sons, Bristol.

ISAACSON, P.G., DOGHAN, A., PRICE, S.K. & SPENCER, J. (1989) Immunoproliferative small intestinal disease. An immunohistochemical study. *Am. J. Surg. Pathol.* **13,** 1023.

KEHRL, J.H., MURAGUCHI, A. & FAUCI, A.S. (1984) Human B cell activation and cell cycle progression: stimulation with anti-μ and *Staphylococcus aureus* Cowan strain I. *Eur. J. Immunol.* **14,** 115.

KIKUTANI, H., INUI, S., SATO, R., BARSUMIAN, E.L., OWAKI, H., YAMASAKI, K., KAISHO, T., UCHIBAYASHI, N., HARDY, R.R., HIRANO, T., TSUNASAWA, S., SAKIYAMA, F., SUEMURA, M. & KISHIMOTO, T. (1986) Molecular structure of human lymphocyte receptor for immunoglobulin E. *Cell*, **47,** 657.

KISHIMOTO, T., MIYAKE, T., NISHIZAWA, Y., WATANABE, T. & YAMAMURA, Y. (1975) Triggering mechanism of B lymphocytes. I. Effect of anti-immunoglobulin and enhancing soluble factor on differentiation and proliferation of B cells. *J. Immunol.* **115,** 1179.

KNAPP, W. (1989) 4th International Conference on human leucocyte differentiation antigens. Vienna, February 21–25. *Tissue Antigens*, **33,** 49.

KUMARARATNE, D.S. & MACLENNAN, I.C.M. (1981) Cells of the marginal zone of the spleen are lymphocytes derived from recirculating precursors. *Eur. J. Immunol.* **11,** 865.

KURITANI, T. & COOPER, M.D. (1982) Human B cell differentiation. II. Pokeweed mitogen-responsive B cells belong to a surface immunoglobulin D negative subpopulation. *J. exp. Med.* **155,** 1561.

LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227,** 680.

LINTON, P.J. & KLINMAN, N.R. (1989) Self-non-self discrimination by precursors of memory B cells. In *Progress in Immunology VII* (ed. by F. Melchers *et al.*) pp. 424. Springer-Verlag, Berlin.

LIU, Y.J., OLDFIELD, S. & MACLENNAN, I.C.M. (1988) Memory B cell in T cell dependent antibody responses colonize the splenic marginal zones. *Eur. J. Immunol.* **18,** 355.

MACLENNAN, I.C.M. & GRAY, D. (1986) Antigen-driven selection of virgin and memory B cells. *Immunol. Rev.* **91,** 61.

MACLENNAN, I.C.M., GRAY, D., KUMARARATNE, D.S. & BAZIN, H. (1982) The lymphocytes of splenic marginal zones: a distinct B-cell lineage. *Immunol. Today*, **3,** 305.

MACMICHAEL, A., BEVERLEY, P.C.L., COBBOLD, S., CRUMPTON, M.J., GILKS, W., GOTCH, F.M., HOGG, N., HORTON, M., LING, N., MACLENNAN, I.C.M., MASON, D.Y., MILSTEIN, C., SPIEGELHALTER, D. & WALDMANN, H. (Eds) (1987) *Leucocyte Typing III.* Oxford University Press, Oxford.

MYHRE, M. & ISAACSON P.G. (1987) Primary B cell gastric lymphoma—a re-assessment of histogenesis. *J. Pathol.* **152,** 1.

NEOH, S.H., GORDON, C., POTTER, A. & ZOLA, H. (1986) The purification of mouse monoclonal antibodies from ascitic fluid. *J. immunol. Methods*, **91,** 231.

OWEN, M.J., KISSONERGHIS, A.M. & LODISH, H.F. (1980) Biosynthesis of HLA-A and HLA-B antigens in vivo. *J. biol. Chem.* **255,** 9678.

PARKER, D.C. (1975) Stimulation of mouse lymphocytes by insoluble anti-mouse immunoglobulin. *Nature*, **258,** 361.

REINHERZ, E.L., HAYNES, B.F., NADLER, L.M. & BERNSTEIN, I.D. (EDS) (1985) *Leucocyte Typing II: Human B Lymphocytes* Springer-Verlag, New York.

SOUHAMI, R.L., BABBAGE, J. & SIGFUSSON, A. (1983) Defective *in vitro* antibody production to varicella zoster and other virus antigens in patients with Hodgkin's disease. *Clin. exp. Immunol.* **53,** 297.

STEIN, H., GERDES, J. & MASON, D.Y. (1982) The normal and malignant germinal centre. *Clinics Haematol.* **11,** 531.

WALDMANN, M.C.H. (1986) Production of monoclonal antibodies. In *Monoclonal Antibodies* (ed. by P. C. L. Beverley) p. 1. Churchill Livingstone, Edinburgh.

WALSH, F.S. & CRUMPTON, M.J. (1977) Orientation of cell surface antigens in the lipid bilayer of lymphocyte plasma membrane. *Nature*, **269,** 307.

ZHANG, J., LIU, Y.J., MACLENNAN, I.C.M., GRAY, D. & LANE, P.J.L. (1988) B cell memory to thymus-independent antigens type 1 and type 2: the role of lipopolysaccharide in B memory induction. *Eur. J. Immunol.* **18,** 1417.